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Characterization of Unclassified Nonfermentative Gram Negative Bacteria in Drinking Water

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CHARACTERIZATION OF UNCLASSIFIED NONFERMENTATIVE GRAM NEGATIVE BACTERIA IN DRINKING WATER

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PUBLISHED PAPER

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ABSTRACT

The group of bacteria most frequently encountered in water distribution systems consists of nonfermentative gram negative rods. Only a small percentage of these bacteria are identifiable using commercially prepared kits or other identification keys because many isolates fail to grow in conventional media used for biochemical characterization. In this study, modified base media were developed and used with specific substrates to detect enzymatic activity. The use of the modified base media combined with a lower incubation temperature allowed expression of increased enzymatic activity by many of the organisms. Although this permitted the characterization of the majority of these organisms, exact identification was unsuccessful since these organisms do not fit descriptions of known species found in current taxonomic schema.

INTRODUCTION

The sanitary quality of drinking water has traditionally been based upon the presence or absence of organisms of fecal origin. The presence of coliform bacteria has been used as an indicator of fecal pollution with the attendant hazard of fecal pathogens. The Standard Plate Count (SPC), which is used to quantify the bacterial content of a given sample has also been used to judge the sanitary quality of potable water. The vast majority of the bacterial isolates from the SPC are nonfermentative gram negative rods. These isolates are quite different from the prolific enteric fermenters and are often dysgonic or scant growing organisms. Intensive studies have been conducted on nonfermentative, gram negative rods isolated from clinical specimens (11-15). These nonfermentative "saprophytes," isolated along with known pathogens, such as Staphylococcus aureus, have until quite recently been ignored by clinicians and medical bacteriologists. The advent of the extensive therapeutic use of immunosuppressive drugs, X-Ray therapy, and various other procedures, which alter the hosts' defense, has caused some of these "saprophytic" organisms to be recognized as clinically important. These organisms, normally innocuous to healthy individuals, become insidious pathogens in debilitated patients. Many of these nonfermentative gram negative bacteria are now recognized as opportunistic pathogens. Opportunistic pathogens may be defined as organisms that cause infectious disease predominantly in people who have impaired local or general natural defense mechanisms, such as patients in hospitals, clinics, nurseries and rest homes (11). Since potable water is used by patients for drinking, sponge baths, and therapeutic whirlpool baths, the use of public water supplies

that contain excessive levels of these organisms is a matter of concern.

The purpose of this investigation was to attempt to identify many of the organisms isolated from drinking water that could not be characterized and speciated by commercial kits or a taxonomic key described by CDC. The significance of the unknown, unclassified aquatic bacteria may be important as other investigators find similar isolates of questionable origin. Use of new project base media, low temperature, and extended incubation were investigated for obtaining better growth and reactions for characterization.

MATERIALS AND METHODS

Two hundred and ninety-four bacterial isolates from the Cincinnati Water Distribution System were studied. These organisms were subcultured from Levine Eosine Methylene Blue Agar (EMB), m-Endo agar, and Standard Plate Count agar, (SPC)(1). The API-20E (Analytab Products, Plainview, New York) was used to identify these isolates and the nonfermenters were further analyzed using a procedure developed by CDC (14).

A second study was also done on bacteria isolated from the Cincinnati Water Distribution System from water samples collected at dead end locations. These samples contained no residual chlorine and the bacterial cultures were isolated from SPC agar. One hundred colonies from the SPC plates were subcultured to fresh SPC plates for purity and also to a new medium, R3A (a modified R2A) used for subculturing of dysgonic bacteria from potable water (D. J. Reasoner and E. E. Geldreich, Abstr. Ann. Meet. Am. Soc. Microbiol. 1979, N7, p. 180).

Since subcultures from SPC agar very often failed to grow upon transfer to fresh SPC agar, the original medium of isolation, subcultured colonies were picked only from the R3A plates to R3A slants to establish a SPC profile.

The subcultures from the R3A slants were inoculated into pairs of O/F glucose as described by Hugh and Leifson (7). Only borosilicate tubes were used since soft glass may leach alkali and seriously alter the pH and sensitivity of the medium. After 24 and 48 hrs of incubation at 35°C, all tubes were observed for fermentation and/or oxidation of glucose, or lack of reaction (glucose nonoxidizers). All isolates were screened by the API 20 E system. For the glucose oxidizers and the glucose non-oxidizers, a second analysis was made following the non-fermentative schema developed by CDC (14).

The selected isolates were divided into three catagories: Category I, glucose fermenters; Category II, glucose oxidizers; and Category III, glucose non-oxidizers. Organisms in Categories I and II were identifiable using conventional procedures. The organisms in Category III were not identifiable by conventional means. These organisms consisting of nonfermenters and nonreactive bacteria (non-glucose oxidizers) were inoculated a second time into O/F glucose media. Single O/F tubes, with no oil overlay, were inoculated from the unknown, nonkeyed isolates and incubated at 30°C instead of traditional 35°C for up to 21 days. Oxidase tests were repeated for isolates from the R3A medium (Oxidase Test Reagent, Marion Scientific Corporation, Kansas City, Missouri 64114) and MacConkey slants were inoculated for growth observance

after 3 days of incubation (Difco Laboratories, Detroit, Michigan 48232). The general procedure is outlined in Figures 1 and 2. In Figure 2, these organisms while not identified, were characterized using the CDC guide and designated as Category III. Characterization was accomplished by observing reactions upon specially modified media listed under media preparation. Incubation for Category III was at 30°C.

For the gram negative diplococci, subcultures to EMB agar were done to observe colonial morphology. For those isolates that appeared as tiny blue colonies on EMB (13), a deoxyribonucleic acid (DNA) transformation was performed to a competent auxotroph of Acinetobacter calcoaceticus (2,8) to verify their tentative identification as Acinetobacter calcoaceticus.

MEDIA PREPARATION

Commercial O/F tubes, triple sugar iron agar slants, SS plates, blood agar plates containing 5% defibrinated sheep blood, and MacConkey slants, were prepared as described (Difco Laboratories, Detroit, Michigan 48232). For the DNase test, commercial Difco medium was used as a base. Reagents and procedures for gram - stains and flagella-stains were prepared as described in Laboratory Methods on Special Medical Bacteriology (2). The basal project medium was modified O/F medium without the pH indicator as follows:

Tryptose	2.0
NaCl	5.0
K_2HPO_4	0.3
Agar	this will vary from 1.0 to 15.0 gms depending on specific substrates
H_2O	1000 ml

For all media, the pH was adjusted as required, brought to boil, and distributed into tubes, capped with regular caps or Permeable Membrane Caps (PM), for respiratory type organisms (Kimble Glass, Owens Illinois, Inc. , P. O. Box 1035, Toledo, Ohio 43666). Sterilization was done by autoclaving at 121°C for 15 minutes, or filtered through 0.2 micron filter into a sterile container. After sterilization, media was poured into plates aseptically. For the tube media, media, the tubes were allowed to solidify upright or slanted as required.

Amino Acids:

L-Ornithine, L-Arginne, or L-Lysine, was added to a concentration of 5 gm/L to the base project medium. In addition, .02 gms brom cresol purple, 1 gm of glucose, 2.0 gms agar were also added. For DL-Amino acids, 10 gms/L was used. The pH was adjusted to 6.5, and the medium was dispensed into 13 x 100 mm tubes, capped with conventional caps and autoclaved. After inoculation, caps were closed tightly to exclude air and the tubes were read daily by comparing to appropriate controls (no amino acid substrate). Glucose nonfermenting organisms will not give acid reactions but were read for alkaline reactions of dark purple.

Bacterial Controls

Ornithine

Pseudomonas putrefaciens +
P. maltophilia -

Arginine

Pseudomonas aeruginosa +
P. maltophilia -

Lysine

Pseudomonas maltophilia +
Salmonella paratyphi A -

Indole Production:

Base media only was used for indole production from tryptone. Upon addition of Kovac's reagent, red color (2) development indicates indole (+) reaction.

Bacterial controls

E. coli, indole +
Klebsiella pneumoniae, indole -

Nitrate Reduction:

Base medium plus 2 gms potassium nitrate, and 1 gm of agar.* Adjust pH 6.8 - 7.2., and dispense 6 ml in 6 x 125 mm tubes containing inverted glass vials for gas. Cap tubes with Permeable Membrane (PM) caps (Kimble Glass) nitrate reduction detected using reagents (A) and (B) (2). First observe inverted vial for gas production.

Reagent A - 8 gm/1000 ml sulfonilic acid/5N acetic acid.
Reagent B - 5 gm/1000 ml alpha naphylamine/5N acetic acid.

Add two drops of each reagent A and B 24 hrs *after* growth, red color positive for nitrite or nitrate reduction.

Bacterial Controls

Aeromonas hydrophila, nitrate +
Pseudomonas pseudomallei, nitrate -

*Category III organisms grow poorly or not at all in liquid media.

Nitrite Reduction:

Base media plus 1 gm of potassium nitrite and 1 gm of agar. Dispense 6 ml into 16 x 125 mm tubes with gas inserts. Use permeable membrane caps (PM). Observe for gas daily.

Bacterial Controls

Alcaligenes denitrificans, gas = +
Alcaligenes faecilis, no gas = -

Tetrazolium Reduction:

Basal medium plus 0.05 gm 2, 3, 5 triphenyl-tetrazolium chloride, and 3.0 gms agar. Adjust pH 6.8 - 7.2. Pour plates and keep right side up for semi-solid media. Inoculate with a single streak across plate.

Bacterial Controls

Pseudomonas aeruginosa, red	=	positive (+)
Citrobacter freundii, colorless	=	negative (-)

For Selenium Reduction:

Basal medium plus 0.1 gm sodium selenite, and 3.0 gm of agar. Adjust pH 6.8 - 7.2. Pour plates, streak through center.

Bacterial Controls

Salmonella typhimurium, red	=	positive (+)
Salmonella paratyphi A, colorless	=	negative (-)

Methylene Blue Reduction:

Basal medium plus 8.0 ml of 0.5% aqueous methylene blue, 2.0 gms of agar and 1.0 gm glucose. Adjust pH between 6.8 to 7.2. Place 4 ml into 13 x 100 mm tubes with PM caps.

Bacterial Controls

Enterobacter aerogenes, colorless	=	methylene positive (+)
Klebsiella pneumoniae, blue	=	methylene blue negative (-)

Motility:

Tryptone 10.9 gms, NaCl 5.0 gms, agar 3.0 gms, 2,3,5 triphenyltetrazolium Cl 0.05 gms, $FeNH_4$ SO_4 0.20 gms, sodium thiosulfate 0.2 gms, distilled water 1000 ml.

Adjust pH 7.0 - distribute into 13 x 100 mm tubes with PM caps, inoculate with a needle, stabbing to 1/2 inch above butt of tube.

Bacterial Controls

Non-motile, H_2S -	=	Red line along stab, Salmonella paratyphi A
Non-motile, H_2S +	=	Black line along stab, Flavobacterium meningosepticum
Motile, H_2S -	=	Diffused pink cloud, E. coli
Motile, H_2S +	=	Black cloud, Salmonella typhimurium

Urea:

Urea solution - urea 2.0 gm, phenol red 0.012 gm*, and water 10 ml. Filter through sterile 0.2 micron membrane into sterile tube. Solution for control, as above, but omit urea.

*For convenience prepare 1.2% solution and add 0.1 ml to 9.9 ml of water.

Basal medium - tryptone 0.2 gm, glucose 0.1 gm, $KH_2 PO_4$ 0.2 gm, agar 1.9 gm, and distilled water 90 ml.

Adjust to pH 6.9, autoclave for 15 minutes at 121°C. Cool to 50-55°C. Aseptically add 10 ml of sterile urea solution as prepared above. Mix, aseptically distribute 3 ml volumes into sterile 13 x 100 mm tubes with PM caps. Slant all tubes until solidified.

Bacterial Controls

Proteus sp. red	=	positive (+)
Salmonella sp. neutral	=	negative (-)

Starch Hydrolysis:

Basal medium plus 2.5 gms starch (wheat), 15.0 gms of agar. Adjust pH 6.8 to 7.2. Prepare pour plates. Inoculate by single streak through center. Starch hydrolysis indicated by a clearing zone adjacent to the streak. Can also be tested by placing a few drops of Gram's iodine on both the clear zone and on the opaque part or edge of plate for control observance.

Bacterial Controls

Aeromonas hydrophila, colorless	=	positive (+)
Pseudomonas aeruginosa, blue	=	negative (-)

Hippurate Hydrolysis:

Basal medium plus 3.0 gms sodium hippurate, 2.5 ml of 4% phenol red and 2.5 gms of agar. Adjust pH 6.8 to 7.2, pour plates (semi-solid agar) do not invert plates.

Bacterial Controls

Serratia marcescens deep red	=	positive (+)
Salmonella typhimurium yellow	=	negative (-)

Gelatin Hydrolysis:

Basal medium plus agar 1.0 gm, glucose 1.0 gm, gelatin 120 gm, pH 6.8 to 7.2. Heat to boiling to melt and distribute in 4 ml amounts in 13 x 100 mm tubes with PM caps. Sterilize at 121°C for 15 min. Inoculate heavily with loop. Read daily for gelatin liquification after cooling below 20°C for 15 minutes.

Bacterial Controls

Serratia marcescens, liquid	=	hydrolysis positive (+)
Escherichia coli, no liquid	=	hydrolysis negative (-)

DNase:

DNase test agar (Difco) 42.0 gm, methyl green 0.05 gm, and H_2O 1000 ml. Adjust pH to 7.5 before adding methyl green because deoxyribonucleic acid (DNA) and methyl green will not combine at a lower pH.

Pour plates, inoculate by single streak through center of plate. Decomposition of polymer attached to methyl green render the methyl green colorless.

Bacterial Controls

Pseudomonas putrifaciens, colorless = DNase positive (+)
Escherichia coli, green = DNase negative (-)

Esculin Hydrolysis:

Basal media plus glucose 1.0 gm, esculin 1.0 gm, ferric citrate 0.5 gm, and agar 15.0 gm. Adjust pH 6.8 to 7.2, prepare slants with PM caps.

Bacterial controls

Klebsiella pneumoniae, reddish black = positive +
Pseudomonas aeruginosa, colorless = negative -

RESULTS AND DISCUSSION

Table 1 shows the results of identification studies conducted on the 294 isolates from the Cincinnati Water Distribution System. The organisms listed in Table 1 include primary pathogens as well as opportunistic pathogens. Among the opportunistic pathogens is *Acinetobacter calcoaceticus*, formerly *Herellea*, considered of little significance a decade ago. This non-fermentative organism, not of fecal origin, is frequently found in water distribution systems and deemed by some clinicians as a primary pathogen (12).

The organisms listed in Table 1 constitute only 15% of the isolates from the Cincinnati Water Distribution System. Of the 100 cultures isolated in the second study in which special efforts to isolate dysgonic cultures were made, 15% could be identified using clinical identification schema. The remaining isolates could not be identified or characterized using taxa of the API or CDC data basis. Most of these isolates when subcultured to SPC, the original isolation medium, failed to grow. The reason for this is unknown. Those same colonies were successfully subcultured upon R3A and stored on R3A for characterization. These isolates, designated as Category III, did not grow on most commercially prepared media, with the exception of O/F media, thus preventing biochemical determination to establish identity. The observance of growth in the O/F medium prompted the use of O/F base, without the indicator, to be used with specific substrates for observation of enzymatic activities or by-products from specific substrates. With the use of these media and a lower temperature of incubation (30°C) characterization was possible based upon comparison with similar gram negative rods from the CDC data base. Major tests used to separate clinical isolates into groups using the CDC key are glucose utilization, growth on MacConkey, and oxidase reaction. Figure 2 shows the number of isolates in each group following the use of these tests. Over half of these isolates could not be characterized because they became nonviable during transfers after isolation. Table 2 is based on the CDC schema for identification of isolates which are glucose oxidizers, MacConkey positive, and oxidase positive at 35°C temperature of incubation. Table 2 shows the biochemical reactions of two distribution system isolates grown at two temperatures (30°C and 35°C). Neither isolate

60 or 62 exhibited carbohydrate oxidation at 35°C, but at 30°C, acid was produced from glucose and mannitol by isolate 60. Isolate 62 utilized glucose, sucrose, and maltose at the lower temperature of 30°C. At 30°C, duration of acid reaction may be extended before the pH reverts to neutrality or becomes alkaline (6). Table 3 shows that no growth was observed with the use of conventional or commercially prepared media. With the use of specifically prepared media, (essentially O/F base without the indicator) growth occurred for all isolates. Positive nitrate and nitrite reduction as well as non-motility was observed with the modified media. Some negative reactions were also observed on the modified media. These negative reactions could not be ascertained on conventional media since no growth occurred. Table 4 shows complementary tests, some of which are recommended by the American Taxonomy Society for new species. Each modified base medium contained a specific substrate. Using the basal project media, isolate 60 reduced methylene blue, hydrolyzed urea, hippurate, and decarboxylated arginine. Isolate 62 showed positive reactions for urea, starch and esculin hydrolysis, and decarboxylation of lysine.

Table 5 shows isolates which were non-reactive on O/F at 35°C, but acidity was produced at 30°C by these isolates. Growth occurred in the project media with the added biochemicals for indole and urea. Urea was hydrolyzed by strain number 20. Table 6 shows the complementary reactions upon various substrates with the project base media. Sometimes the project media did not support growth, designated as ng (no growth) for these dysgonic (scant growing) organisms.

Table 7 shows those isolates that did not utilize the glucose even at 30°C incubation. They grew on MacConkey agar and were oxidase positive. Complementary test results for these four isolates are shown in Table 8. The four isolates showed consistent reduction patterns, with the exception of isolate number 3 which failed to reduce nitrate. A consistent pattern of hydrolysis was also observed. All isolates hydrolyzed hippurate and failed to hydrolyze the other substrates. No definite pattern was observed for the amino acids. All isolates gave negative reactions for indole and DNase. Motility was observed for all four of the isolates. Table 9 (CDC Table 11) lists the profile of Mima (Acinetobacter) polymorpha, currently designated as Acinetobacter calcoaceticus, Pseudomonas maltophila, and Bordetella parapertussis. These organisms are under the general grouping of glucose nonoxidizers, MacConkey positive, and oxidase negative. Part of Category III organisms appearing in Tables 10 and 11 are also under this general grouping and are characterized using modified media containing the specific substrates and incubated at 30°C. Isolates 22 and 23 are gram negative diplococci, which were non-reactive with the API method, but showed distinct colonial morphology (tiny blue) on EMB agar (6). A deoxyribonucleic acid (DNA) transformation was completed to a competent auxotroph of Acinetobacter calcoaceticus (2,8). This reaction enabled positive identification of isolates 22 and 23 as A. calcoaceticus. Ordinarily, Acinetobacter calcoaceticus will subculture to SPC. In this case, however, it was not sub-cultured in SPC agar but was recovered on R3A medium showing the advantage of this technique to recover perhaps a stressed strain of Acinetobacter since it was also non-reactive in the commercial kit. Numbers 59 and 61, also gram negative diplococci, were negative for DNA transformation to a competent auxotroph of A. calcoaceticus. The test

was done because of cellular morphology (gram negative diplococci in stationary phase) and negative oxidase test ruled out Neisseria. No distinct morphology of tiny blue colonies was observed on EMB with isolates 59 and 61.

Tables 12 and 13 list those gram negative rods which are glucose nonoxidizers, MacConkey negative, and oxidase positive. Characterization was done with these isolates using the modified media and 30°C incubation. An acid fast organism, number 9, was recovered using this technique.

SUMMARY

This study has provided insight into the myriad types of nonfermentative gram negative bacteria in drinking water. Specialized media for cultivation of these microorganisms and a system for their characterization were developed.

The identification of these organisms is a tedious task, the values of which must be weighed against the potential public health risk posed by their presence in potable water. The presence of these non-classified organisms, non-subcultural except to R3A, are of little concern today as Herellea was yesterday. The consequences may become serious to the debilitated taking whirlpool therapy. The significance of these dysgonic organisms may lie in the near future as information is exchanged between the environmental and clinical microbiologists.

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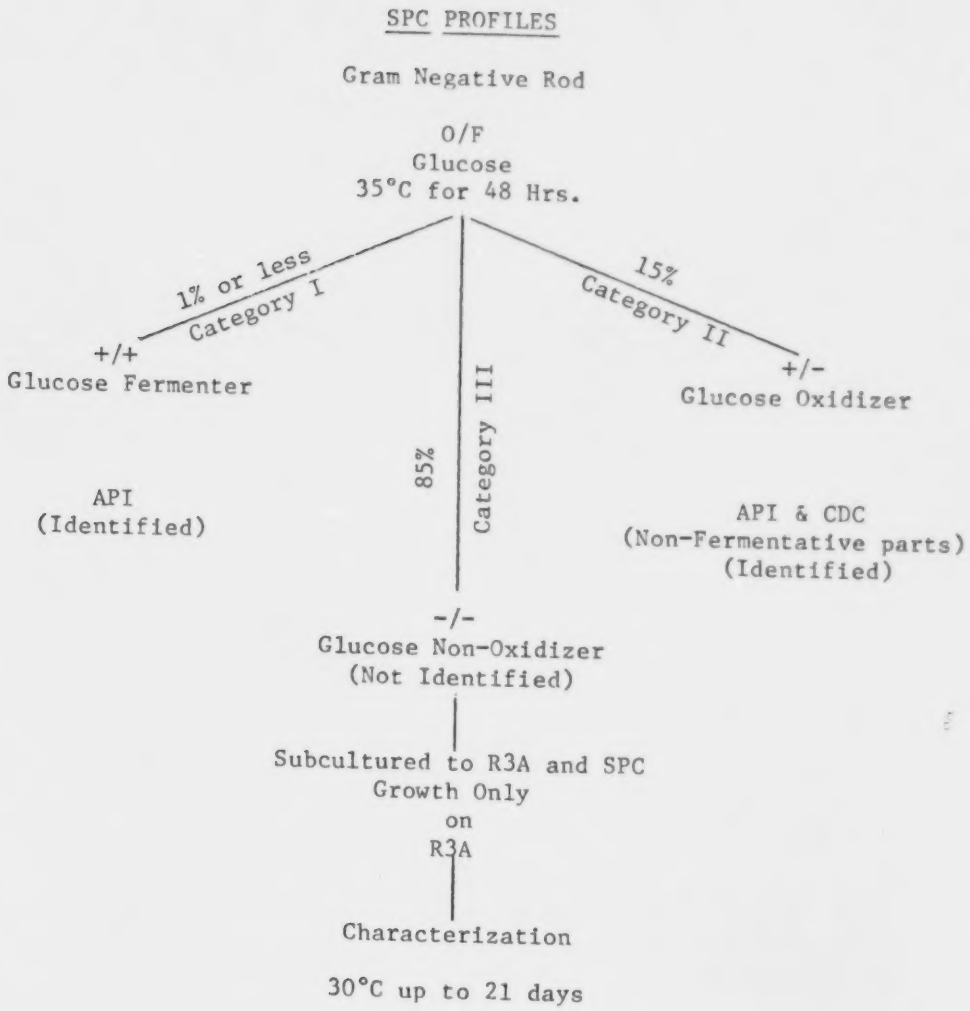

FIGURE 1.

Flow Diagram for Identification and Characterization

Incubation 30°C

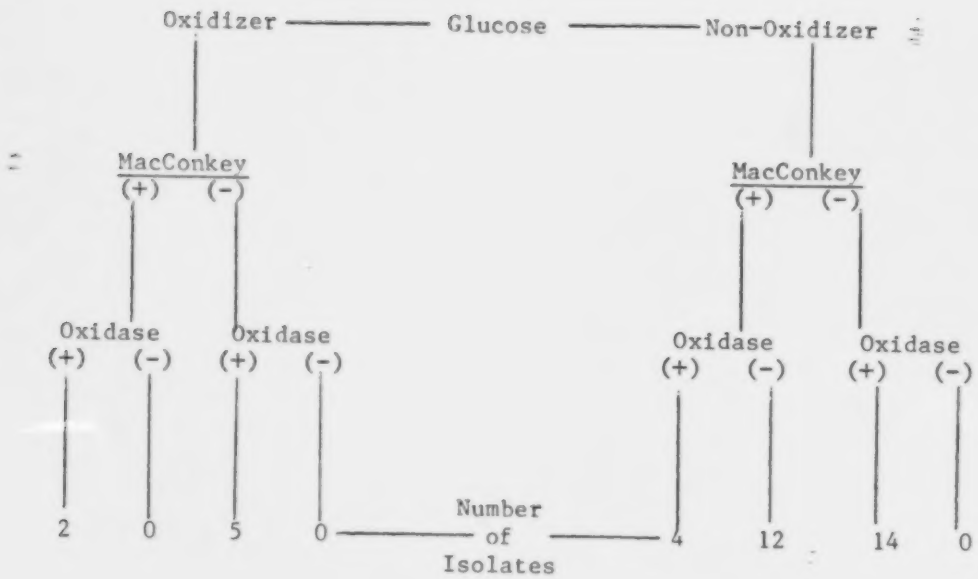

FIGURE 2

Category III Organisms - Subculture recovery only upon R3A. Split into groups according to Glucose, MacConkey and Oxidase reactions.

TABLE 1

BACTERIAL SPECIES ISOLATED FROM CINCINNATI DRINKING WATER DISTRIBUTION SYSTEMS

Gram Positive	Gram Negative	Acid Fast
Staphylococcus coagulase positive (a)	Fermenters	*Mycobacterium sp*
Staphylococcus coagulase negative	*Escherichia coli*	
Bacillus sp	*Klebsiella pneumoniae*	
	Enterobacter cloacae	
	Enterobacter sakazakii	
	Enterobacter agglomerans	
	Citrobacter freundii	
	Chromobacterium violaceum (b)	
	Serratia marcescens (b)	
	Aeromonas hydrophila (b)	
	Nonfermenters	
	Pseudomonas aeruginosa (b)	
	Pseudomonas fluorescens	
	Pseudomonas maltophilia (b)	
	Moraxella sp	
	Acinetobacter calcoaceticus (b)	
	Neisseria flavescens	
	Alcaligenes sp	

(a) Primary Pathogen
(b) Opportunistic Pathogens
Acinetobacter & *Aeromonas* sometimes considered primary pathogens by some clinicians.

TABLE 2

CARBOHYDRATE REACTIONS OF GRAM NEGATIVE
OXIDIZERS OF GLUCOSE
MACCONKEY POSITIVE OXIDASE POSITIVE

	Glucose	Xylose	Mannitol	Lactose	Sucrose	Maltose
At 35°C - No Reaction						
60	N	N	N	N	N	N
62	N	N	N	N	N	N
At 30°C - Reaction at lower temperature						
60	A/N	B	A	B	B	B
62	A/N	B	B	B	$A \pm / N$	A^{4+}

N = Neutral (Non-Reactive)
A = Acid Production
B = Basic Reaction

TABLE 3

GRAM NEGATIVE OXIDIZERS OF GLUCOSE
MACCONKEY POSITIVE, OXIDASE POSITIVE

	Indole	Gas from Nitrate	Test for nitrite formation	Complete reduction of nitrate & nitrite	Growth on SS	Lysine decar-boxylase	Arginine dihy-drolase	Motility	Flagella
At 35°C									
60	ng	ng	ng	ng	ng	ng	ng	ng	ND
62	ng	ng	ng	ng	ng	ng	ng	ng	ND
At 30°C - With specially developed basal project media									
60	-	+	+	+	* ng	-	+	G NM	ND
62	-	-	+	-	ng	+	-	G NM	ND
Exception									

* SS media, commercially prepared, not modified.

ng = No Growth
NM = Non-Motile
ND = Not Done
G = Growth

TABLE 4

COMPLEMENTARY TESTING - SPECIAL PROJECT MEDIA

Isolate Number	Reduction			Hydrolysis					Amino Acids Decarboxylase Dehydrolase				
	Tetrazolium	Methylene Blue	Selenium	Urea	Starch	Esculin	Hippurate	Gelatine	Control	Ornithine	Arginine	Lysine	DNase
60	-	+	-	+	-	-	+	-	-	-	+	-	-
62	-	-	-	+	+	+	-	-	-	-	-	+	-

TABLE 5
GRAM NEGATIVE OXIDIZERS OF GLUCOSE
MACCONKEY NEGATIVE, OXIDASE POSITIVE

30°C

Isolate	Indole	Nonsoluble Pigment R3A	Glucose	Xylose	Mannitol	Lactose	Sucrose	Maltose	Urea	
2	-	Yellow	A	A/B	A/B	B	B	A	-	Non-Motile
4	-	Yellow	A/N	A/N	B	B	B	B	-	Non-Motile
8	-	Yellow	A/B	A/N	B	B	B	B	-	Non-Motile
20	-	Yellow	A	A	B	A	B	A	+	Non-Motile
48	-	Colorless	A	A	B	N	A	N	-	Non-Motile

TABLE 6

COMPLEMENTARY TESTING SPECIAL PROJECT MEDIA

30°C

Test	Isolate Number				
	2	4	8	20	48
Reduction					
Nitrate	+	+	-	+	-
Nitrite	-	-	-	-	-
Tetrazolium	+	+	ng	ng	+
Methylene Blue	+	+	+	-	-
Selenium	+	+	+	+	+
Hydrolysis					
Urea	-	-	-	+	-
Starch	-	-	-	+	+
Hippurate	+	+	+	-	+
Gelatin	-	-	-	-	+
Esculin	+	+	+	-	+
Amino Acids					
Control	-	-	-	-	-
Ornithine	+	+	-	+	-
Arginine	+	+	-	+	+
Lysine	-	-	-	+	-
Other					
Indole	-	-	-	-	-
DNase	+	-	-	+	+
Motility	-	-	-	-	-

TABLE 7

GRAM NEGATIVE GLUCOSE NONOXIDIZERS (NONFERMENTERS)
MACCONKEY POSITIVE, OXIDASE POSITIVE, AEROBIC

30°C

	Flagella	Nitrate Reduction	Gas from nitrate	Gas from nitrite nitrate not reduced	H_2S, butt of TSI	OF Xylose	Growth on SS	Urea
3	1-2 Polar	-	-	-	-	B	-	-
12	1-2 Polar	+	-	-	-	B	-	-
40	1-2 Polar	+	-	-	-	B	-	-
45	1-2 Polar	+	-	-	-	B	-	-

TABLE 8

COMPLEMENTARY TESTING SPECIAL PROJECT MEDIA

30°C

Test	Isolate Number			
	3	12	40	45
Reduction				
Nitrate	-	+	+	+
Nitrite	-	-	-	-
Tetrazolium	+	+	+	+
Methylene Blue	+	+	+	+
Selenium	+	+	+	+
Hydrolysis				
Urea	-	-	-	-
Starch	-	-	-	-
Hippurate	+	+	+	+
Gelatin	-	-	-	-
Esculin	-	-	-	-
Amino Acids				
Control	-	-	-	-
Ornithine	-	-	-	+
Arginine	+	-	-	+
Lysine	-	+	-	+
Other				
Indole	-	-	-	-
DNase	-	-	-	-
Motility	+	+	+	+

TABLE 7

GRAM NEGATIVE GLUCOSE NONOXIDIZERS (NONFERMENTERS)
MACCONKEY POSITIVE, OXIDASE POSITIVE, AEROBIC

30°C

	Flagella	Nitrate Reduction	Gas from nitrate	Gas from nitrite nitrate not reduced	H_2S, butt of TSI	OF Xylose	Growth on SS	Urea
3	1-2 Polar	-	-	-	-	B	-	-
12	1-2 Polar	+	-	-	-	B	-	-
40	1-2 Polar	+	-	-	-	B	-	-
45	1-2 Polar	+	-	-	-	B	-	-

TABLE 10

GRAM NEGATIVE GLUCOSE NONOXIDIZERS (NONFERMENTERS)
MACCONKEY POSITIVE, OXIDASE NEGATIVE
30°C

	Urea	Soluble pigment	OF Maltose	Gelatin	Motility	"Beta" Hemolysis	
10	-	orange -	A ±	-	-	- green	
11	-	orange	-	-	-	- green	
15	ng	orange -	-	+	-	green	
22	±	buff -	-	-	-	- (-) cocci	A. calcoaceticus
23	-	buff	-	-	-	- (-) cocci	A. calcoaceticus
24	-	bright orange	A +/ B	-	-	green	
28	-						
33	-	bright orange	-	-	-	±	
35	-	orange -	-	ng	-	-	
39	+	buff	B	+	-		
59	-	yellow -	A + / N	ng	-	-	- cocci
61	-	orange	-	-	-	-	- cocci

TABLE 11

COMPLEMENTARY TESTING SPECIAL PROJECT MEDIA

30°C

Test	Isolate Number												
	10	11	13	15	22	23	24	28	33	35	39	59	61
Reduction													
Nitrate	-	+	Non-Viable	-	-	+	+	-	-	-	+	+	-
Nitrite	-	-	"	-	-	-	+	+	-	-	-	-	-
Tetrazolium	-	-	"	+	+	+	+	+	+	+	+	-	-
Methylene Blue	-	-	"	-	-	+	-	-	-	-	+	+	-
Selenium	+	+	"	+	+	+	+	+	-	+	+	+	-
Hydrolysis													
Urea	-	-	"	±	-	-	-	+	+	-	+	-	-
Starch	-	+	"	+	-	-	-	+	+	-	+	-	+
Hippurate	-	-	"	-	+	+	-	-	+	-	+	+	-
Gelatin	+	+	"	+	-	-	-	-	+	-	+	-	+
Esculin	+	+	"		-	-	±	+	±	+	+	+	+
Amino Acids													
Control	-	-	"	-	-	-	-	-	-	-	-	-	-
Ornithine	-	-	"	-	-	-	-	-	-	-	+	+	-
Arginine	-	-	"	-	-	-	+	-	-	-	-	+	-
Lysine	-	-	"	-	-	-	-	-	-	-	+	-	+
Other													
Indole													
DNase	+	+	"	+	-	-	-	+	-	-	-	+	+
Motility	-	-	"	-	-	-	-	-	-	-	-	-	-

TABLE 12

GRAM NEGATIVE GLUCOSE NONOXIDIZERS (NON-FERMENTERS)
MACCONKEY NEGATIVE, OXIDASE POSITIVE

Positive No.	Indole	Urea	30°C OF Xylose	Reduction of nitrate	Notes
1	-	-	A+/N	+	Acid from Xylose feebly
5	-	-	A^{2+}	+	
6	-	-	B	-	Creamy
(Acid Fast) 9	-	+	A^{2+}	-	+ Rod orange pigment
14	-	+	N	-	Pink colonies
21	-	+	N	-	Orange colonies - diplococci-void-centers
25	-	-	N	-	Orange colonies
26	-	-	N	-	Orange colonies
29	-	+	N	-	Light orange-pigment
34	-	-	N	-	Orange
36	-	+	N	-	Orange
47	-	-	N	-	Orange - (Key 10?)
52	-	+	N	+	Light pink
58	-	-	N	-	Pink

TABLE 13

COMPLEMENTARY TESTING SPECIAL PROJECT MEDIA

30°C

Test	Isolate Number													
	1	5	6	9	14	21	25	26	29	34	36	47	52	59
Reduction														
Nitrate	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Nitrite	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tetrazolium	+	+	+	+	+	+	+	+	±	+	-	+	-	-
Methylene Blue	-	+	-	+	-	-	-	+	-	-	-	-	-	-
Selenium	+	+	+	+	+	-	+	+	+	-	+	+	+	+
Hydrolysis														
Urea	-	-	-	+	+	+	-	-	±	-	+	-	+	-
Starch	+	-	-	+	+	-	+	-	+	+	+	+	+	+
Hippurate	-	+	-	-	-	-	-	+	+	-	-	-	-	-
Gelatin	-	-	+	±	-	-	-	-	-	+	+	-	+	-
Esculin	+		+	-	-		+	-	+	+	+	+	+	+
Amino Acids														
Control	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Ornithine	+	-	-	+	-	+	-	-	-	-	-	-	-	-
Arginine	+	+	-	+	-	+	-	-	-	-	-	-	+	-
Lysine	+	-	-	+	-	+	-	-	-	-	-	-	-	-
Other														
Indole	-	-		-	-	-	ng	ng	-	-	-	-	-	-
DNase	+	-	+	-	-	-	+	-	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-